Enhancement of Antibody Detection in Cancer Using Panel of Recombinant Tumor-associated Antigens


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Abstract
Cancer sera contain antibodies which react with a unique group of autologous cellular antigens called tumor-associated antigens (TAAs). This study determines whether a mini-array of multiple TAAs would enhance antibody detection and be a useful approach to cancer detection and diagnosis. The mini-array of TAAs comprised full-length recombinant proteins expressed from cDNAs encoding c-myc, p53, cyclin B1, p62, Koc, IMP1, and survivin. Enzyme immunoassay was used to detect antibodies in 527 sera from six different types of cancer. Antibody frequency to any individual TAA was variable but rarely exceeded 15–20%. With the successive addition of TAAs to a final total of seven antigens, there was a stepwise increase of positive antibody reactions up to a range of 44–68%. Breast, lung, and prostate cancer patients showed separate and distinct profiles of reactivity, suggesting that uniquely constituted antigen mini-arrays might be developed to distinguish between some types of cancer. Distinct antibody profiles were not observed in gastric, colorectal, and hepatocellular carcinomas with this set of seven TAAs. Detection of autoantibodies in cancer can be enhanced by using a mini-array of several TAAs as target antigens. Additional studies in early cancer patients and high-risk individuals and the design of unique antigen panels for different cancers would help to determine whether multiple antigen mini-arrays for the detection of autoantibodies might contribute a clinically useful noninvasive approach to cancer detection and diagnosis.

Introduction
It is a well-established observation that autoimmunity can be associated with cancer, and one of the forms of its expression is the development of antibodies to autologous cellular antigens. One of the most extensively studied cancer-associated antigens is p53, a tumor suppressor protein. Autoantibodies to p53 in cancer were first reported in 1982 (1), and since then, there have been numerous reports confirming and extending this finding (reviewed in Ref. 2). The types of cellular proteins which induce autoantibody responses are quite varied and include oncogene products, such as HER-2/neu and ras (3); cellular proteins, which shield mRNAs from natural physiological degradation, such as p62 (4) and CRD-BP (5); onconeural antigens in the paraneoplastic disorder syndromes (6, 7); and differentiation antigens, such as tyrosinase and the cancer/testis antigens (8).

Factors leading to the production of such autoantibodies are not completely understood, but the available data show that many of the target antigens are cellular proteins whose aberrant regulation could lead to tumorigenesis, such as p53, HER-2/neu, and ras (3); cellular proteins, which shield mRNAs from physiological degradation, such as p62, and cell cycle control proteins, such as cyclin B1 (9, 10). A previous study showed that lung tumors contained several types of p53 gene mutations, including missense, stop codon, and frame shift mutations, but it was the missense mutations resulting in overexpression of protein with altered function and increased stability that correlated with antibody production (11). In the case of p62, which is a fetal protein absent in adult tissues, immunogenicity appears to be related to abnormal expression of p62 in tumor cells (12), and with the onconeural antigens in paraneoplastic neurological disorders, antibody responses are thought to be related to ectopic expression in tumor cells of normally neuron-restricted cellular proteins (6, 7). The immune system in certain cancer patients appears to have the capability of sensing these abnormalities, and we have proposed that autoantibodies might be regarded as reporters identifying aberrant cellular mechanisms in tumorigenesis (13).

Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence of these autoantibodies in normal individuals and noncancer conditions. Enthusiasm for this approach has been tempered by low sensitivity. We showed recently (14) in a study using 777 sera from different types of cancer that autoantibodies to p62 were detected in 11.6 ± 1.15% and antibodies to Koc (15), another TAA, in 12.2 ± 1.17%. However, when the data were analyzed for...
frequency of antibodies to either p62 or Koc, the frequency rose to 20.5 ± 1.45%. These initial observations were the basis for the current study using several hundred sera from six different types of cancer to determine reactivities to a mini-array of seven selected TAAs. We show that the frequency of antibodies to any individual antigen rarely exceeded 15–20%, but with the successive addition of antigens to the panel, there was stepwise increase in the percentage of positive reactors to between 44 and 68% against a combined panel of seven antigens. In addition, breast, lung, and prostate cancers showed separate and distinctive profiles of antibody responses, suggesting that tailor-made TAA panels could be developed for different cancers and that TAA mini-arrays might provide another approach to tumor detection and diagnosis.

Materials and Methods
Patients and Sera. The serum bank of the Tumor Cell Engineering Laboratory of Xiamen University, Fujian Province, People’s Republic of China, has a collection of sera from cancer patients which has been used for epidemiological and other studies, and this laboratory made sera available from 321 patients with different types of cancer, including 64 with breast cancer, 56 with lung cancer, 45 with colorectal cancer, 91 with gastric cancer, and 65 with HCC. As control, sera were obtained from 82 individuals from the same region who were having annual health examinations and had no evidence of malignancy. Sera from 206 prostate cancer patients treated at Loma Linda University Medical Center (Loma Linda, CA) were also available for this study. As comparison for the normal human sera from China, 264 sera from the San Diego area were available and came from a large epidemiological and genetic study of hemochromatosis, in which these “normal” sera were obtained from the nonhospitalized adult San Diego population (courtesy of Dr. E. Beutler, The Scripps Research Institute). In addition, as representative of patients with known immune reactivity to other autologous cellular antigens, sera from 62 patients with SLE and 41 patients with SS were available from the serum bank of the Autoimmune Disease Center (Scripps) and were evaluated with the same immunoassays for their response to TAAs. This study was approved by the Institutional Review Boards of the respective academic centers.

Expression and Purification of Recombinant TAAs. Seven antigens, c-myc, p53, cyclin B1, p62, Koc, IMP1, and survivin were selected for the expression of recombinant proteins. Recombinant p62 has been expressed from a clone derived from a cDNA expression library by immunoscreening with antibody from a patient with HCC (4). p62 cDNA was subcloned into pET28a vector, producing a fusion protein with NH2-terminal 6x histidine and T7 epitope tags. The recombinant protein expressed in Escherichia coli BL21 (DE3) was purified using nickel column chromatography. Koc cDNA cloned in the pcDNA3 vector (15) was similarly subcloned to pET28a vector, and recombinant protein was expressed as above. IMP1 construct pCMV5-IMPI was kindly provided by F.C. Nielsen (16) and p53 clone (p53S3N) by Yuxin Yin of Columbia University (New York, NY) and subcloned into pET28a for protein expression. cDNA from c-myc was amplified by PCR from human fetal liver tissue and survivin cDNA from human survivin expressed sequence tag clone (BQ258433) before subcloning in pET28a vector. Recombinant cyclin B1 had been prepared and used previously (9) and was isolated from a pGEX construct expressing cyclin B1 with glutathione S-transferase fusion partner. The expression of adequate amounts of recombinant protein was examined in SDS-PAGE, and Coomassie Blue staining was used to determine that expression products of expected molecular sizes were produced. In addition, Western immunoblot analysis was used to confirm that the bands seen in SDS-PAGE were reactive with reference antibodies. The antibodies used were rabbit polyclonal anti-IMPI from F.C. Nielsen (16), anti-Koc/IMP3 from F. Muller-Pillasch (15), which were raised against specific COOH-terminal peptides of the respective proteins, and anti-p62, which was raised against the full-length protein (4). Reactivities of p53, c-myc, and cyclin B1 were determined with monoclonal antibodies obtained from Oncogene Research Products (Boston, MA). Rabbit polyclonal antiserum against the COOH-terminal peptide was obtained from ProSci, Inc. (Poway, CA).

ELISA. Purified recombinant proteins were diluted in PBS to a final concentration of 0.5 μg/ml, and 200 μl were pipetted into each well to coat Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, VA). All human sera were diluted 1:200, incubated with antigen-coated wells at room temperature for 90 min followed by washing with PBS, and developed with horseradish peroxidase-conjugated goat antihuman IgG (Caltag Laboratories, San Francisco, CA) using the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, Germany). Additional details of this procedure have been described previously (4, 14). Each serum sample was tested in duplicate, and the average A492 nm was used for data analysis.

The cutoff value designating positive reactions was conventionally chosen as the mean absorbance of the 82 normal Chinese human sera + 3 SD. Because several hundred test sera were analyzed at different time periods, each run of ELISA included 10 NHS samples and 2 positive control samples. The 10 NHS samples represented a range above and below the mean of the 82 Chinese normals, and the average value of the 10 normals was used in each run to normalize all absorbance values to the standard mean of 82 normal. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. χ2 tests were initially used to determine whether the frequencies of positive sera in each cohort of cancer patients was significantly higher than in the normal cohort; statistical significance at α levels 0.05 and 0.01 are reported. Estimated sensitivities and specificities are given with associated SE.

Cancer sera were also tested against four cellular antigens: (a) dsDNA; (b) Sm; (c) SS-A/Ro; and (d) SS-B/La that are known antigenic targets of autoantibodies in the prototype autoimmune diseases, SLE, and SS (17). Although they are known to be autoantigens in systemic rheumatic diseases, cancer sera have not generally been evaluated for antibodies to these antigens, and this analysis could help to determine the extent of specificity of TAAs in cancer. Enzyme immunoassay kits from Helix Diagnostics (West Sacramento, CA) were used for these analyses.

Results
Frequency of Antibodies to Panel of TAAs and Range of Antibody Titers. Several parameters were considered to be important in the design of this study. The TAAs were selected to include onconeplast product c-myc and tumor suppressor gene product p53. Cyclin B1 has been reported to be antigenic in humoral and cell-mediated immune responses in HCC and epithelial cell tumors, respectively (9, 10), P62 (4), Koc (15), and IMP1 (16) are three proteins containing similar RNA-binding motifs, and their mRNAs are either overexpressed in cancer (5, 15), or antibodies have been identified to the protein products (4, 14). Survivin is a cellular protein that is antiapo-
Table 1  Frequency of antibodies to seven cancer-associated antigens

<table>
<thead>
<tr>
<th>Cancer</th>
<th>No. tested</th>
<th>c-myc</th>
<th>p53</th>
<th>Cyclin B1</th>
<th>p62</th>
<th>Koc</th>
<th>IMP1</th>
<th>Survivin</th>
<th>Any of seven Ags</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>64</td>
<td>12 (18.8)**</td>
<td>5 (7.8)</td>
<td>3 (4.7)</td>
<td>5 (7.8)</td>
<td>9 (14.1)**</td>
<td>5 (7.8)</td>
<td>5 (7.8)</td>
<td>28 (43.8)**</td>
</tr>
<tr>
<td>Lung</td>
<td>56</td>
<td>6 (10.7)**</td>
<td>9 (16.1)**</td>
<td>15 (26.8)**</td>
<td>12 (21.4)**</td>
<td>5 (8.9)*</td>
<td>4 (7.1)</td>
<td>6 (10.7)**</td>
<td>38 (67.9)**</td>
</tr>
<tr>
<td>Colorectal</td>
<td>45</td>
<td>2 (4.4)</td>
<td>8 (17.8)**</td>
<td>7 (15.6)**</td>
<td>5 (11.1)*</td>
<td>4 (8.9)*</td>
<td>6 (13.3)*</td>
<td>2 (4.4)</td>
<td>23 (51.1)**</td>
</tr>
<tr>
<td>Gastric Ca</td>
<td>91</td>
<td>14 (15.4)**</td>
<td>12 (13.2)**</td>
<td>14 (15.4)**</td>
<td>8 (8.8)*</td>
<td>17 (18.7)**</td>
<td>15 (16.5)**</td>
<td>9 (9.9)*</td>
<td>48 (52.7)**</td>
</tr>
<tr>
<td>HCC Ca</td>
<td>65</td>
<td>16 (24.6)**</td>
<td>7 (10.8)*</td>
<td>8 (12.3)*</td>
<td>8 (12.3)**</td>
<td>9 (13.8)**</td>
<td>10 (15.4)**</td>
<td>7 (12.5)*</td>
<td>37 (56.9)**</td>
</tr>
<tr>
<td>Total (China)</td>
<td>321</td>
<td>50 (15.6)**</td>
<td>41 (12.8)**</td>
<td>47 (14.6)**</td>
<td>38 (11.8)**</td>
<td>44 (13.7)**</td>
<td>40 (12.5)**</td>
<td>29 (9.0)*</td>
<td>174 (54.2)**</td>
</tr>
<tr>
<td>Prostate (U.S.)</td>
<td>206</td>
<td>8 (3.9)</td>
<td>10 (4.9)</td>
<td>29 (14.1)**</td>
<td>52 (25.2)**</td>
<td>19 (9.2)**</td>
<td>18 (8.7)*</td>
<td>6 (2.9)</td>
<td>95 (46.1)**</td>
</tr>
<tr>
<td>NHS (China)</td>
<td>82</td>
<td>0 (0.0)</td>
<td>2 (2.4)</td>
<td>2 (2.4)</td>
<td>1 (1.2)</td>
<td>1 (1.2)</td>
<td>2 (2.4)</td>
<td>2 (2.4)</td>
<td>9 (11.0)</td>
</tr>
<tr>
<td>NHS (U.S.)</td>
<td>264</td>
<td>4 (1.5)</td>
<td>3 (1.1)</td>
<td>5 (1.9)</td>
<td>6 (2.3)</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>26 (9.9)</td>
</tr>
<tr>
<td>SLE (U.S.)</td>
<td>62</td>
<td>3 (4.8)</td>
<td>0</td>
<td>1 (1.6)</td>
<td>1 (1.6)</td>
<td>1 (1.6)</td>
<td>2 (3.2)</td>
<td>1 (1.6)</td>
<td>3 (4.8)</td>
</tr>
<tr>
<td>SS (U.S.)</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a  Cutoff value: mean + 3 SD of NHS (China). Ps between cancer and NHS were calculated to be <0.05 (*) or 0.01 (**).
serum. If there was significant increased frequency of coordinate expression of two antibodies, this would be represented as higher frequency (percentage) in the bar graphs shown in Fig. 3. There appeared to be minor increases in frequencies of coexpressed antibodies, such as anti-c-myc and anti-p53 in breast cancer, anti-c-myc and anti-IMP1 in gastric cancer, and anti-c-myc and anti-IMP1 in HCC. These increases did not reach statistical significance, and in general, no clear coexpres-
sition of antibodies was observed. This finding supported the data in Table 2 showing that the sequential addition of antigens resulted in the detection of new antibodies, leading to the increased likelihood of detecting anti-TAA activity.

**Immunoreactivity of Cancer Sera with Other Cellular Autoantigens.** It was important to establish that autoantibodies to TAAs were not because of general immunoreactivity. Table 3 shows that reactivity with other cellular antigens, such as dsDNA, Sm, SS-A/Ro, and SS-B/La, was rarely found in cancer sera, but in SLE sera, they were in the expected and frequencies documented previously (17). There was reactivity in one or two cancer sera of 184 sera from five different types of cancers with a similar occasional reactivity in normal human sera, but these were unlike the higher frequencies observed in SLE sera.

**Discussion**

The most comprehensive studies on autoantibodies to TAAs have been on p53, and it was of interest to compare our data with those reported previously. Of the six different cancers in this study, the lowest positive rate was 4.9 ± 1.2% for prostate cancer, and the highest was 17.8 ± 2.13% for colorectal cancer. In a compilation of anti-p53 studies (2), the rate for prostate cancer was 2.7%, and it was 24.6% for colorectal cancer. The positive rates for breast, lung, gastric cancers, and HCC were intermediate between prostate and colorectal cancers in the review and this study. In our study, the positive rate for prostate cancer was not statistically different from the rate for normal human sera, and the same conclusion was found in previous studies, whereas the positive rates for breast, lung, colorectal, gastric cancers, and HCC were significantly different from normal controls in this and other studies (2, 20). This agreement with studies documented previously supports the accuracy of the immunoassay method used in this study.

This study points to an interesting feature of tumor immunity, that with an increasing number of antigens in a multiantigen immunoassay, the likelihood of detecting antibody in any cancer serum increased from ~15 to 26% when one antigen was used to 44–68% when seven antigens were used. Earlier studies that might have anticipated these results involved the use of a two-antigen panel (14) or was restricted to one type of cancer (21) in contrast to this study using seven known TAAs.
and several hundred patients with six different types of cancer. The sensitivity of the seven-antigen panel immunoassay system varied for different types of cancer. The factor or factors leading to these differences have not been examined, but from Fig. 2, it appeared that there was a stronger association between certain antigens and some cancers. In prostate cancer, p62 and cyclin B1 appear to be distinctly more reactive antigens than c-myc, p53, or survivin. In lung cancer, cyclin B1, p62, and p53 appear to be the more reactive antigens, whereas in breast cancer, they are c-myc and Koc. In HCC, c-myc stands out compared with other antigens which are about equal in antigenicity. A clear association of highly reactive antigens with colorectal and gastric cancer is not apparent. However, this initial study is based on seven TAAs, and the selection of antigens, although based on their known association with tumors, is by no means optimized and could be further refined with panels designed for greater specificity for certain types of cancer. There is support at the genetic level for this notion. Gene abnormalities, such as mutations or overexpression, tend to occur in combinations that vary from tissue to tissue (22), and if autoimmune responses represent immune system reactions to abnormal gene expression (13), one might expect differences in autoantibody profiles from one type of tissue cancer to another. Mini-arrays of TAAs could be constructed to include among others, several other known TAAs, such as HER-2/neu and ras, certain cell cycle proteins, and antigens involved in cell-mediated immune reactions, many of which might have concomitant humoral immune responses (8, 23).

The judicious selection of antigens to be included in panels or arrays of TAAs is extremely important because not all cellular proteins recognized as antigens by cancer sera are cancer specific. This was especially apparent in studies in HCC.
where it was possible to analyze sera from the same HCC patients many months or years preceding malignancy when the patients had liver cirrhosis, chronic hepatitis, and autoimmune liver disease. Some of the autoantibodies identified with sera obtained when HCC was diagnosed were already present before the development of malignancy (24, 25), and these included antibodies against nucleolar antigens fibrillarin, nucleolar organizer region 90 protein, and B23, all of which are also recognized by antibodies from scleroderma and other autoimmune diseases. This feature has also been reported using SEREX technology (26), in which sera from colon cancer patients used in immunoscreening cDNA expression libraries identified several autoantigens associated previously with noncancer disorders, including hydroxymethylglutaryl-17, poly ADP ribose polymerase, and U1 small nuclear ribonucleoprotein, as well as cellular protein PINCH associated with aging and recognized by antibodies in normal human sera (27). The definition of TAA can probably be best determined by studies of significant numbers of cancer, as well as noncancer disorders.

Recently, an autoantigen array has been used for characterization of autoantibodies in patients with autoimmune rheumatic diseases, demonstrating potential applications, including the possibility of early diagnosis and characterization of specificity (28). Our study was not designed to show the predictive potential of autoantibodies as tumor markers but to report the novel finding of increasing sensitivity for detecting autoantibodies in cancer when larger panels of cancer-associated antigens are used. Nevertheless, cancer-associated antigen panels might conceivably be used for early detection of tumors in high-risk individuals. The appearance of antibodies to TAA has been demonstrated in HCC in serum samples during antecedent liver disease (23, 29, 30). Anti-p53 antibodies were detected in two heavy smokers before clinical detection of lung cancer, and in 1 patient, early treatment resulted in good response, which correlated with total disappearance of p53 antibodies (31, 32). Anti-p53 antibodies were detected in workers exposed to vinyl chloride before clinical detection of angiosarcoma (33), and anti-HER-2neu antibodies can be detected in patients with early stage breast cancer (34). Anti-p53 antibodies have been detected in Barrett’s esophagitis (35), a condition often precluding esophageal cancer and in betel nut chewers with oral leukoplakia (36), a condition precluding oral malignancy. Important future studies would be to determine whether mini-arrays of antibody markers might be useful in identifying early cancer in high-risk individuals. Furthermore, additional efforts should be aimed at increasing both the sensitivity and specificity of antibodies as cancer markers by expanding antigen arrays to include antigens which might be more selectively associated with some tumors and not with others.

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